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Enrichment of proteinase activity in deteriosomes, a new class of microvesicles

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Non-sedimentable microvesicles, termed deteriosomes, have been isolated from the cytosol of bean cotyledons by ultrafiltration, and found to be enzymatically active. Specifically, they possess proteinase activity that is able to catabolize exogenous proteins as well as deteriosome proteins. The proteolytic activity is inhibited by heat-denaturation and known proteinase inhibitors (iodoacetate and phenylmethylsulfonyl fluoride), and the pattern of deteriosome-associated proteinase activity changes with advancing senescence of the cotyledon tissue. Deteriosomes are formed by blebbing from membranes. The finding that they possess proteinase activity is consistent with an earlier proposal [(1991) Proc. Natl. Acad. Sci. USA 88, 2269–2273] that they are involved in membrane turnover and serve as a vehicle for moving bilayer-destabilizing phospholipid and protein catabolites out of membranes into the cytosol for further processing. The data also indicate that a significant proportion of the proteinase activity traditionally considered to be cytosolic is associated with deteriosomes.

Proteinase, Microvesicle; Senescence; *Phaseolus vulgaris*

1. INTRODUCTION

Microvesiculation is an inherent feature of membrane translocation and assembly. Plasma membrane-specific proteins, for example, become associated with the endoplasmic reticulum through co-translational insertion and are subsequently translocated through the Golgi apparatus to the plasmalemma in microvesicles [2,3]. Recently, a new class of microvesicles that appears to be involved in membrane turnover and degradation has been isolated and partially characterized. These microvesicles have been termed deteriosomes and are distinguishable from those involved in membrane translocation in that they are non-sedimentable and enriched in phospholipid and protein catabolites [1]. Specifically, they contain lower levels of phospholipid than membranes do, and are enriched relative to membranes in phospholipid catabolites, in particular free fatty acids, and in low molecular weight polypeptides.

Deteriosomes are isolated by ultrafiltration (1,000 kDa cut-off filter) of the cytosol (post-microsomal supernatant) and are present in both plant and animal tissues [1,4]. They appear to be formed by blebbing from membrane surfaces and can also be generated in vitro from isolated membranes under conditions in which phospholipid catabolism is activated [1]. Recent data support the notion that deteriosomes serve as a vehicle for moving bilayer-destabilizing phospholipid and protein catabolites out of membranes into the cytosol for further processing. It has been demonstrated, for example, that gel phase-forming phospholipid catabolites are present in deteriosomes and are removed from membranes during deteriosome formation [5]. Indeed, these lipid catabolites accumulate in membranes in senescing tissue causing a lateral phase separation in the bilayer, and this has been correlated with progressively impaired deteriosome formation as senescence progresses [5].

The present study demonstrates that deteriosomes are able to catabolize exogenous proteins as well as deteriosome proteins, and that the pattern of deteriosome-associated proteinase activity changes with advancing senescence. The finding that deteriosomes exhibit proteinase activity is consistent with the notion that they are involved in membrane turnover and dissolution, and further suggests that part of the proteinase activity in plant tissue previously thought to be soluble is in fact associated with non-sedimentable microvesicles in the cytosol.

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2. MATERIALS AND METHODS

2.1. *Plant materials and fractionation procedures*

Bean seeds (*Phaseolus vulgaris* L. cv Kinghorn) were germinated in moist vermiculite at 29°C and 90% relative humidity under conditions of etiolation. Young and senescing cotyledons were harvested after 2 and 7 days of growth, respectively, and fractionated to obtain microsomal membranes, deteriosomes and deteriosome-free cytosol. The tissue was homogenized (20% w/v) in 50 mM $NaHCO_3$, 300 mM sucrose, pH 7.0, for 45 s in an Omnimixer, and for an additional 1 min in a Polytron homogenizer at 4°C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 10,000 × *g* for 20 min. The supernatant was centrifuged at 130,000 × *g* for 1 h to

pellet microsomal membranes. The microsomal membranes were washed by resuspending the pellet in homogenizing buffer and pelleted again by centrifugation at 130,000 × *g* for 1 h

Deteriosomes were isolated as described previously [1]. The post-microsomal supernatant (130,000 × *g*, 1 h) was centrifuged at 250,000 × *g* for 12 h to sediment any residual membrane Deteriosomes and deteriosome-free cytosol were isolated by passing this final supernatant through a 1,000,000 Da cut-off filter. (Identical results were obtained using a 300,000 Da cut-off filter.) Residual cytosolic contamination in the separated deteriosome fraction was reduced to less than 2% by washing with isolation buffer (50 mM $NaHCO_3$, 300 mM sucrose, pH 7 0) using the same filter Cytosolic proteins trapped within the deteriosomes were released by permeabilizing the bilayers with 0 1% Triton X-100 [6] and washing the treated deteriosomes three times with isolation buffer In some experiments, deteriosomes were further purified by fractionation on a Superose-12 column (40 × 2 0 cm) as described previously [1]. For this purpose, the washed deteriosomes (4 mg protein equivalents) were loaded onto the column and eluted with 50 mM $NaHCO_3$, 300 mM sucrose, pH 7.0

2.2. *Assay procedures*

Proteinase activity in each of the separated fractions was visualized by SDS-PAGE of trichloroacetic acid (TCA)-insoluble polypeptides [7]. For this purpose, microsomal membranes, deteriosomes and deteriosome-free cytosol (20 μg protein equivalent) were incubated for up to 3 h at 29°C in 200 μl of assay buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 0.25 mM $CaCl_2$ (40 μM free Ca^{2+} [8]), pH 7.0) containing, when added, 60 μg bovine serum albumin (BSA). The reaction was stopped by adding 100 μl of solubilizing buffer (0 25 M Tris-HCl, pH 6.8, containing 4% SDS, 10% mercaptoethanol, 20% glycerol, 0.015% Bromophenol blue) and boiling for 5 min. The polypeptides were fractionated on 12% (or in some experiments 18%) SDS-PAGE gels and visualized by staining with Coomassie brilliant blue.

Proteinase activity was also determined quantitatively by measuring the release of TCA-soluble peptides [9,10]. Microsomal membranes, deteriosomes and deteriosome-free cytosol (100 μg protein equivalent) were incubated for up to 3 h at 29°C in 1 ml of assay buffer (see above) containing, when added, 600 μg bovine serum albumin (BSA). The reaction was stopped by addition of cold 5% (final concentration) TCA. The resulting protein precipitate was pelleted by centrifugation (10,000 × *g*, 5 min), and the peptides in the supernatant were quantified by measuring absorbance at 280 nm [9,11] or by using ninhydrin [10]. Proteinase activity at different pH values was measured in the same way except that 100 mM phosphate rather than Tris-HCl was used as the buffer.

Deteriosomes were heat-denatured by placing them in boiling water for 10 min. For proteinase inhibitor studies, deteriosomes were preincubated with the inhibitors (1 mM iodoacetic acid or 1 mM phenylmethylsulfonyl fluoride (PMSF)) for 30 min at 29°C before the reaction was started by the addition of BSA. Protein was measured as described by Bradford [12] using BSA as a standard. Fatty acids were analyzed according to Morrison and Smith [13].

3. RESULTS AND DISCUSSION

Deteriosomes proved capable of catabolizing BSA, an exogenous substrate used previously for plant protease measurements [7,14], more effectively than did either membranes or deteriosome-free cytosol. Two pronounced TCA-insoluble polypeptide catabolites of BSA with apparent molecular weights of 44 and 52 kDa, respectively, were formed during a 3 h reaction period by deteriosomes from young 2-day-old cotyledons (Fig. 1A, lane 7). These polypeptides were not detectable when deteriosomes were incubated for a

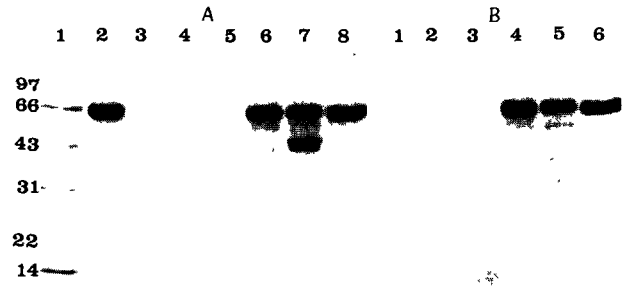

Fig. 1. Visualization of BSA polypeptide catabolites by SDS-PAGE. (A) Polypeptide catabolites formed over a 3 h period by microsomal membranes, deteriosomes and deteriosome-free cytosol isolated from young 2-day-old cotyledons. Lane 1, protein molecular weight (kDa) standards; lane 2, 4 μg BSA; lanes 3–5, 1.3 μg of microsome, deteriosome and deteriosome-free cytosol protein, respectively; lanes 6–8, 1.3 μg of microsome, deteriosome and deteriosome-free cytosol protein, respectively, and 4 μg of BSA. (B) Polypeptide catabolites formed by microsomal membranes, deteriosomes and deteriosome-free cytosol isolated from senescing 7-day-old cotyledons Lanes 1 3, 1.3 μg of microsome, deteriosome and deteriosome-free cytosol protein, respectively, lanes 4–6, 1.3 μg of microsomes, deteriosome and deteriosome-free cytosol protein, respectively, and 4 μg of BSA.

comparable period in the absence of BSA (Fig. 1A, lane 4); nor were they discernable when comparable concentrations (on a protein basis) of microsomal membranes or deteriosome-free cytosol from young 2-day-old cotyledons were allowed to react with BSA over a 3 h period (Fig. 1A, lanes 6 and 8). The 44 and 52 kDa polypeptides resolved by SDS-PAGE appear to be catabolites of BSA inasmuch as they are not detectable for any of the cell-free fractions in the absence of the exogenous substrate (Fig. 1A, lanes 3–5), and they can be attributed to proteinase(s) that is more active in deteriosome fractions than in microsomal membranes or deteriosome-free cytosol (Fig. 1A, lanes 6–8).

Similar data were obtained when microsomal membranes, deteriosomes and deteriosome-free cytosol from senescing 7-day-old cotyledons were tested, except that different catabolites of BSA were found. Specifically, in the presence of 7-day-old deteriosomes, three polypeptide catabolites of BSA with apparent molecular weights of 34, 41, and 56 kDa were formed that were not detectable in the absence of BSA (Fig. 1B, lanes 2 and 5). These distinguishable patterns of catabolite formation suggest that there are senescence-related changes in deteriosome proteinase. The same polypeptide catabolites were also formed, although not nearly to the same degree, by 7-day-old microsomal membranes (Fig. 1B, lanes 1 and 4). This is consistent with the contention that deteriosomes are formed by blebbing from membrane surfaces [1]. Catabolites of BSA were not, however, formed at detectable levels by 7-day-old deteriosome-free cytosol (Fig. 1B, lanes 3 and 6). The data in Fig. 1 were obtained by fractionating the polypeptides on 12% SDS-PAGE gels. No additional BSA-derived polypeptides were obtained when the pro-

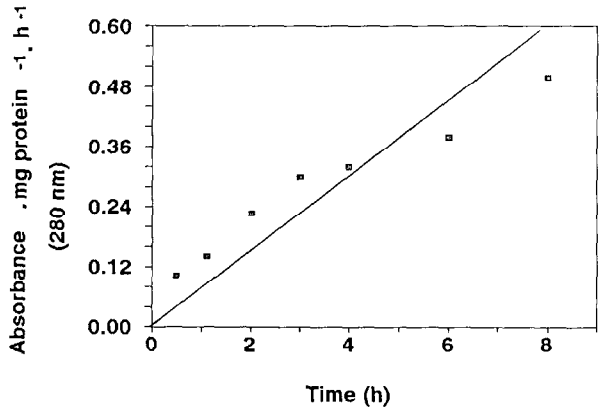

Fig. 2. Time-dependent release of TCA-soluble peptides from BSA by deteriosomes isolated from 2-day-old cotyledons. Values for samples containing no BSA were subtracted. The data are for one of three separate experiments showing the same results.

teins were fractionated on 18% gels run to 2/3 completion (data not shown).

Quantitation of proteinase activity by measuring the release of TCA-soluble peptides from BSA also indicated that deteriosomes exhibit higher activity than do either microsomal membranes or deteriosome-free cytosol. When deteriosomes were incubated in buffer for 3 h in the absence of BSA, the cumulative formation of

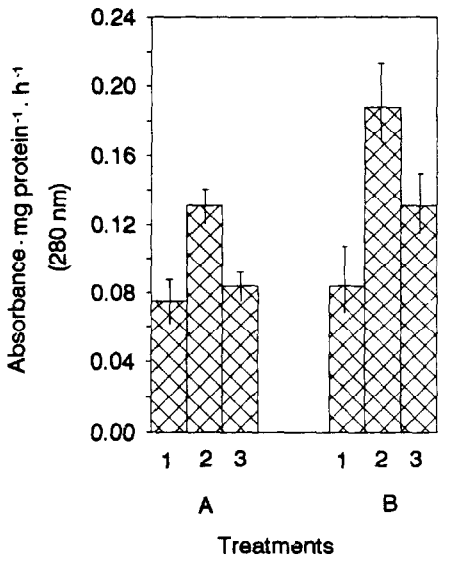

Fig. 3. Quantitation of TCA-soluble peptides formed by microsomal membranes, deteriosomes and deteriosome-free cytosol. (A) Subcellular fractions (1, microsomes; 2, deteriosomes; 3, deteriosome-free cytosol) isolated from young 2-day-old cotyledons incubated for 3 h in the presence of BSA. (B) Subcellular fractions (1, microsomes; 2, deteriosomes; 3, deteriosome-free cytosol) isolated from senescent 7-day-old cotyledons incubated for 3 h in the presence of BSA. Means ± S.E.M. for $n = 3$ are indicated. Values for samples containing no BSA were subtracted. Similar results were obtained using ninhydrin to quantify the TCA-soluble peptides. When the corresponding TCA precipitate was analyzed on 12% SDS-PAGE gels, results identical to those shown in Fig. 1 were obtained.

TCA-soluble peptides ($A_{280} = 0.151 \pm 0.012$ · mg protein^{-1} · h^{-1}; $n = 3$) reflecting proteolytic action on native proteins that was ~ 3-fold higher than that obtained for microsomal membranes ($A_{280} = 0.045 \pm 0.008$ · mg protein^{-1} · h^{-1}; $n = 3$). In the presence of BSA there was a further increase in TCA-soluble peptide formation specifically attributable to catabolism of the exogenous substrate. The release of TCA-soluble peptides rose progressively with time up to 8 h (Fig. 2), and deteriosome-mediated catabolism of BSA was again higher than microsome- or deteriosome-free cytosol-mediated catabolism of the exogenous substrate (Fig. 3A). (The data in Fig. 3 were obtained by measuring changes in A_{280}; comparable data were obtained using ninhydrin.) The pH optimum for TCA-soluble peptide formation from BSA was 6.5 for 2-day-old deteriosomes and 7.0 for corresponding microsomal membranes (Fig. 4). The catabolism of BSA into TCA-soluble peptides was greater for both deteriosomes and deteriosome-free cytosol from senescent 7-day-old cotyledons than for corresponding fractions from young 2-day-old cotyledons (Fig. 3A and B). Deteriosome proteinase activity proved to be largely heat-denaturable and sensitive to PMSF and iodoacetate, known protease inhibitors [15,16], for both young and senescing tissues (Fig. 5A and B). Deteriosomes formed in vitro from isolated microsomal membranes under conditions in which phospholipid catabolism is activated [1] also exhibited BSA-specific protease activity detectable by SDS-PAGE of TCA-insoluble polypeptides as well as the release of TCA-soluble peptides (data not shown).

The contention that deteriosome protease activity is not attributable to cytosolic contamination was confirmed by further purification of the deteriosome preparation on a Superose-12 column. The proteins and fatty acids of washed deteriosomes from 2-day-old cotyledons co-eluted in the void volume during chromato-

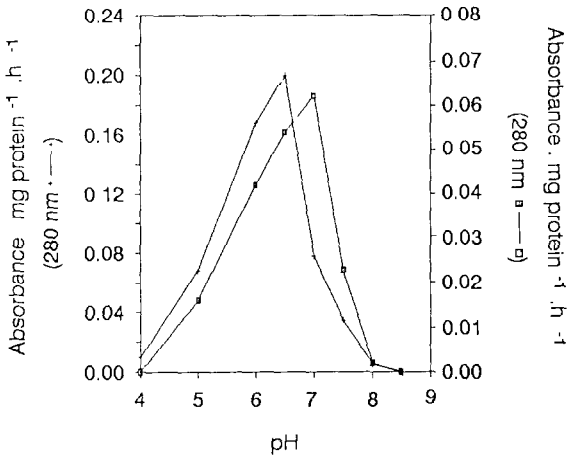

Fig. 4. pH profiles for proteinase activity in deteriosomes (+) and microsomal membranes (□) isolated from 2-day-old cotyledon tissue. Values for samples containing no BSA were subtracted. The data are for one of three separate experiments showing the same results.

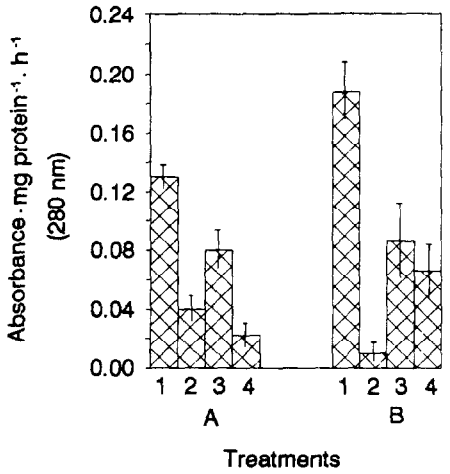

Fig. 5. Effects of heat-denaturation and protease inhibitors on the formation over a 3 h period of TCA-soluble peptides from BSA by deteriosomes isolated from young 2-day-old cotyledons (A) and senescent 7-day-old cotyledons (B). Bars: 1, deteriosomes incubated with BSA; 2, heat-denatured deteriosomes incubated with BSA; 3, deteriosomes incubated with BSA in the presence of 1 mM PMSF; 4, deteriosomes incubated with BSA in the presence of 1 mM iodoacetate. Means ± S.E.M. for $n = 3$ are indicated. Values for samples containing no BSA were subtracted. (Similar results were obtained using ninhydrin to quantify the TCA-soluble peptides.)

graphic fractionation on Superose-12, indicating that they are assembled in a macromolecular complex (Fig. 6A). In addition, protease activity measured as the release of BSA-specific TCA-soluble peptides was detectable in all of the fractions in which protein and fatty acid co-eluted (Fig. 6B), and the fractions obtained from the Superose-12 column also released the 44 and 52 kDa polypeptide catabolites of BSA that were formed by corresponding unfractionated deteriosomes (Fig. 6C). As well, treatment of deteriosomes with 0.1% Triton X-100, a detergent that disturbs lipid bilayers and renders them leaky [6], did not release proteinase activity (data not shown).

It is thus apparent that deteriosomes possess proteinase(s) that is able to catabolize exogenous substrate as well as deteriosome-associated proteins. It has been demonstrated by freeze-fracture electron microscopy that deteriosomes are bilayered in nature [1], and the proteinase is presumably in the bilayer and able to access BSA substrate external to the microvesicles. Other experiments [4] have indicated, however, that deteriosome bilayers are leaky, and thus translocation of BSA into the interior of the microvesicles is not precluded. Inasmuch as deteriosomes are present in a post- microsomal supernatant, the subcellular fraction that has traditionally been considered to represent the cytosol, it would appear that some of the proteolytic activity previously ascribed to the cytosol and thought to be soluble is, in fact, associated with these non-sedimentable microvesicles. The specific activity of proteinase(s) measured as the release of TCA-soluble peptides (with BSA as a substrate) was 1.6-fold and 1.4-fold higher in the deteriosome fraction than in the deteriosome-free cytosol fraction for young and senescent tissue, respectively (Fig. 3). In terms of BSA-specific proteinase activity per g of cotyledon tissue, the deteriosome activity comprised 70 ± 4.3% (S.E.M. for $n = 3$) of the total activity in the post-microsomal supernatant for young 2-day-old tissue and 69 ± 9.9% (S.E.M. for $n = 3$) of the total activity in the post-microsomal supernatant for senescent 7-day-old tissue. Thus, for this tissue at least, the major proportion of the putative cytosolic (soluble) activity is associated with non-sedimentable microvesicles in the cytosol.

Several lines of evidence support the contention that the proteinase activity detectable in deteriosomes is not attributable to cytosolic contamination. First, the specific activity was higher for deteriosomes than for de-

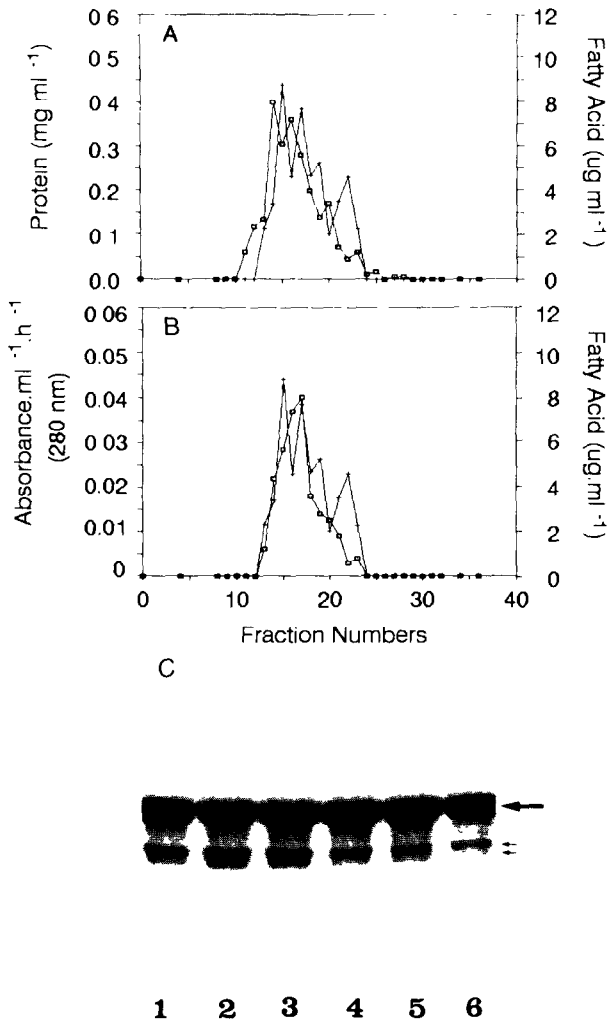

Fig. 6. Chromatographic fractionation of deteriosomes isolated from 2-day-old cotyledons on Superose-12. (A) Protein (□) and fatty acid (+) (B) Proteinase activity (□) and fatty acid (+) (C) Proteinase activity measured by visualization of TCA-insoluble polypeptides from BSA. Lanes 1–6 are fractions 14–19, respectively. Thick arrow denotes BSA; thin arrows denote 52 kDa and 44 kDa polypeptides, respectively

teriosome-free cytosol; indeed, proteolytic fragments of BSA resolvable by SDS-PAGE were not detectable for deteriosome-free cytosol. Second, the level of proteinase activity present in the deteriosome fraction accounted for 70% of the total proteinase activity in the post-microsomal supernatant, yet deteriosome-free cytosol remaining in the deteriosome fraction was reduced to < 2% by successive washes with isolation buffer. Third, permeabilization of deteriosomes with 0.1% Triton X-100 did not release proteinase activity. Finally, purification of deteriosomes by Superose-12 column chromatography, which would separate deteriosomes from any free cytosolic protein, and washing the deteriosomes with 3 M NaBr to remove contaminating cytosolic proteins associated ionically with the microvesicles, did not alter the proteinase activity.

Deteriosome formation appears to be prompted by phospholipid catabolites that promote blebbing from the membrane surface [1]. This, together with the finding that they exhibit proteolytic activity, raises the possibility that deteriosome formation is an integral feature of membrane turnover allowing removal of bilayer-destabilizing protein and lipid catabolites out of the membrane environment into the cytosol for further processing. Indeed, the finding that deteriosomes are themselves capable of carrying out such processing suggests that they have organelle-like properties.

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